PROCESSES FOR PRODUCING A FERMENTATION PRODUCT

REFERENCE TO A SEQUENCE LISTING

This application contains a Sequence Listing in computer readable form. The computer readable form is incorporated herein by reference.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to processes for production of a fermentation product from milled starch-containing material, such as granular starch, at a temperature below the initial gelatinization temperature of the milled starch-containing material in the presence of glucoamylase.

Description of Related Art

Grains, cereals or tubers of plants contain starch. The starch is in the form of microscopic granules, which are insoluble in water at room temperature. When an aqueous starch slurry is heated, the granules swell and eventually burst, dispersing the starch molecules into the solution. During this "gelatinization" process, there is a dramatic increase in viscosity. Because the solids level in a typical industrial process is around 30-40%, the starch has to be thinned or "liquefied" so that it can be handled. This reduction in viscosity is generally accomplished by enzymatic degradation in a process referred to as liquefaction. During liquefaction, the long-chained starch is degraded into smaller branched and linear chains of glucose units (dextrins) by an alpha-amylase.

A conventional enzymatic liquefaction process may be carried out as a three-step hot slurry process. The slurry is heated to between 80-85°C and thermostable alpha-amylase added to initiate liquefaction. The slurry is then jet-cooked at a temperature between 105-125°C to complete gelatinization of the slurry, cooled to 60-95°C and, generally, additional alpha-amylase is added to finalize hydrolysis. The liquefaction process is generally carried out at pH between 5 and 6. Milled and liquefied whole grains are known as mash.

During saccharification, the dextrins from the liquefaction are further hydrolyzed to produce low molecular sugars DP_{1-3} that can be metabolized by a fermenting organism, such as yeast. The hydrolysis is typically accomplished using glucoamylase, alternatively or in addition to glucoamylases, alpha-glucosidases and/or acid alpha-amylases can be used. A full saccharification step typically last up to 72 hours, however, it is common only to do a pre-saccharification of, e.g., 40-90 minutes at a temperature above 50°C, followed by a complete

saccharification during fermentation in a process known as simultaneous saccharification and fermentation (SSF).

Fermentation is performed using a fermenting organism, such as yeast, which is added to the mash. Then the fermentation product is recovered. For ethanol, e.g. fuel, potable, or industrial ethanol, the fermentation is carried out, for typically 35-60 hours at a temperature of typically around 32°C. When the fermentation product is beer, the fermentation is carried out, for typically up to 8 days at a temperature of typically around 14°C

Following fermentation, the mash may be used, e.g., as a beer, or distilled to recover ethanol. The ethanol may be used as, e.g., fuel ethanol, drinking ethanol, and/or industrial ethanol.

It will be apparent from the above discussion that the starch hydrolysis in a conventional process is very energy consuming due to the different temperature requirements during the various steps.

U.S. Patent No. 4,316,956 provides a fermentation process for conversion of granular starch into ethanol.

European Patent No. 140410 provides an enzyme composition for starch hydrolysis.

WO 2004/081193 concerns a method of producing high levels of alcohol during fermentation of plant material. The method includes i) preparing the plant material for saccharification, ii) converting the prepared plant material to sugar without cooking, and iii) fermenting the sugars.

The object of the present invention is to provide improved processes for conversion of milled starch-containing material, such as granular starch, into a fermentation product, such as ethanol.

SUMMARY OF THE INVENTION

The present invention provides processes of producing a fermentation product from starch-containing material without gelatinization of said starch-containing material using glucoamylase.

In the first aspect, the invention provides a process for producing a fermentation product from milled starch-containing material comprising:

(a) saccharifying milled starch-containing material with a glucoamylase having an amino acid sequence shown in SEQ ID NO: 2, or a glucoamylase being at least 70% identical thereto, at a temperature below the initial gelatinization temperature of said starch-containing material,

(b) fermenting using a fermenting organism.

Steps (a) and (b) may be carried out sequentially or simultaneously.

Preferably, a slurry comprising water and milled starch-containing material is prepared before step (a). The dry solid content (DS) lies in the range from 20-55 wt.-%. In order to expose more surface of the starch-containing material it is milled. In an embodiment the particle size is between 0.05-3.0 mm, or at least 30% of the milled starch-containing material fit through a sieve with a 0.05 to 3.0 mm screen. The process of the invention may be carried out for a period of 1 to 250 hours. The pH during saccharification and/or fermentation may be in the range from between 3 and 7. During fermentation the glucose concentration may be kept at a level of below about 3 wt.-%. In a preferred embodiment saccharification and fermentation is carried out simultaneously. According to a preferred embodiment the glucoamylase is derived from a strain of *Athelia*, preferably a strain of *Athelia rolfsii*. The glucoamylase is present in an amount of 0.001 to 10 AGU/g DS. In a preferred embodiment an acid alpha-amylase is present as well. The acid alpha-amylase may be a fungal or bacterial alpha-amylase, preferably a fungal alpha-amylase derived from a strain of *Aspergillus*, especially *A. niger or A. oryzae*. The acid alpha-amylase may be present in a concentration of 0.1 to 10 AFAU/g DS. The ratio between acid alpha-amylase and glucoamylase may be between 0.1 and 10 AGU/AFAU. Optionally the fermentation product, such as ethanol, is recovered after fermentation. Other ingredients and enzyme activities may also be present during the process of the invention. Examples of other enzyme activities are xylanase, cellulase, and phytase activity.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides processes for producing a fermentation product from starch-containing material without gelatinization of said starch-containing material. In one embodiment only a glucoamylase is needed during saccharification and fermentation. According to the invention the desired fermentation product, such as ethanol, can be produced without liquefying the aqueous slurry containing the starch-containing material. If the aqueous slurry containing starch-containing material is heated to above the gelatinization temperature liquefaction is necessary. In general a process of the invention includes saccharifying milled starch-containing material below the gelatinization temperature in the presence of a glucoamylase having the sequence shown in SEQ ID NO: 2, or homologues thereto, to produce sugars that can be fermented into the desired fermentation product by a suitable fermenting organism.

The inventors have found that when producing ethanol from uncooked milled corn using the glucoamylase derived from *Athelia rolfsii* shown in SEQ ID NO: 2 a significantly higher ethanol yield is obtained compared to a corresponding process using glucoamylase

derived from *Aspergillus niger* or *Talaromyces emersonii*. When adding fungal acid alpha-amylase from *Aspergillus niger* (SEQ ID NO: 3) to the process the performance is still significantly higher, i.e., compared to a corresponding process using the *Aspergillus niger* glucoamylase and fungal acid alpha-amylase derived from *Aspergillus niger*.

Accordingly, in the first aspect the invention relates to a process for producing a fermentation product from milled starch-containing material comprising:

(a) saccharifying milled starch-containing material with a glucoamylase having an amino acid sequence shown in SEQ ID NO: 2, or a glucoamylase being at least 70% identical thereto, at a temperature below the initial gelatinization temperature of said starch-containing material,

(b) fermenting using a fermenting organism.

Steps (a) and (b) of the process of the invention may be carried out sequentially or simultaneously.

Before step (a), a slurry of starch-containing material, such as granular starch, having 20-55 wt.-% dry solids, preferably 25-40 wt.-% dry solids, more preferably 30-35% dry solids of starch-containing material may be prepared. The slurry may include water and/or process waters, such as stillage (backset), scrubber water, evaporator condensate or distillate, side stripper water from distillation, or other fermentation product plant process water. Because the process of the invention is carried out below the gelatinization temperature and thus no significant viscosity increase takes place high levels of stillage may be used if desired. In an embodiment the aqueous slurry contains from about 1 to about 70 vol.-% stillage, preferably 15-60% vol.-% stillage, especially from about 30 to 50 vol.-% stillage.

The milled starch-containing material may be prepared by milling starch-containing material to a particle size of 0.05 to 3.0 mm, preferably 0.1-0.5 mm. After being subjected to a process of the invention at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or preferably at least 99% of the dry solids of the starch-containing material is converted into a soluble starch hydrolysate.

The process of the invention is conducted at a temperature below the initial gelatinization temperature. Preferably the temperature at which step (a) is carried out is between 30-75°C, preferably between 45-60°C.

In a preferred embodiment, steps (a) and (b) are carried out as a simultaneous saccharification and fermentation process. In such preferred embodiment the process is typically carried at a temperature between 28°C and 36°C, such as between 29°C and 35°C, such as between 30°C and 34°C, such as around 32°C. According to the invention the temperature may be adjusted up or down during fermentation.

In an embodiment simultaneous saccharification and fermentation is carried out so that the sugar level, such as glucose level, is kept at a low level such as below about 3 wt.-%, preferably below about 2 wt.-%, more preferred below about 1 wt.-%., even more preferred below about 0.5%, or even more preferred below about 0.1 wt.-%. Such low levels of sugar can be accomplished by simply employing adjusted quantities of enzyme and fermenting organism. A skilled person in the art can easily determine which quantities of enzyme and fermenting organism to use. The employed quantities of enzyme and fermenting organism may also be selected to maintain low concentrations of maltose in the fermentation broth. For instance, the maltose level may be kept below about 0.5 wt.-% or below about 0.2 wt.-%.

The process of the invention may be carried out at a pH in the range between 3 and 7, preferably from 3.5 to 6, or more preferably from 4 to 5.

Starch-containing materials

Any suitable starch-containing starting material, including granular starch, may be used according to the present invention. The starting material is generally selected based on the desired fermentation product. Examples of starch-containing starting materials, suitable for use in the processes of present invention, include tubers, roots, stems, whole grains, corns, cobs, wheat, barley, rye, milo, sago, cassava, tapioca, sorghum, rice peas, beans, or cereals, sugar-containing raw materials, such as molasses, fruit materials, sugar, cane or sugar beet, potatoes, and cellulose-containing materials, such as wood or plant residues. Contemplated are both waxy and non-waxy types of corn and barley.

The term "granular starch" means raw uncooked starch, i.e., starch in its natural form found in cereal, tubers or grains. Starch is formed within plant cells as tiny granules insoluble in water. When put in cold water, the starch granules may absorb a small amount of the liquid and swell. At temperatures up to 50°C to 75°C the swelling may be reversible. However, with higher temperatures an irreversible swelling called "gelatinization" begins. Granular starch to be processed may be a highly refined starch quality, preferably at least 90%, at least 95%, at least 97% or at least 99.5% pure or it may be a more crude starch containing material comprising milled whole grain including non-starch fractions such as germ residues and fibers. The raw material, such as whole grain, is milled in order to open up the structure and allowing for further processing. Two milling processes are preferred according to the invention: wet and dry milling. In dry milling whole kernels are milled and used. Wet milling gives a good separation of germ and meal (starch granules and protein) and is often applied at locations where the starch hydrolysate is used in production of

syrups. Both dry and wet milling is well known in the art of starch processing and is equally contemplated for the process of the invention.

The starch-containing material is milled in order to expose more surface. In an embodiment the particle size is between 0.05 to 3.0 mm, or so that at least 30%, preferably at least 50%, more preferably at least 70%, even more preferably at least 90% of the milled starch-containing material fit through a sieve with a 0.05 to 3.0 mm screen, preferably 0.1-0.5 mm screen.

The term "initial gelatinization temperature" means the lowest temperature at which gelatinization of the starch commences. Starch heated in water begins to gelatinize between 50°C and 75°C; the exact temperature of gelatinization depends on the specific starch, and can readily be determined by the skilled artisan. Thus, the initial gelatinization temperature may vary according to the plant species, to the particular variety of the plant species as well as with the growth conditions. In the context of this invention the initial gelatinization temperature of a given starch-containing material is the temperature at which birefringence is lost in 5% of the starch granules using the method described by Gorinstein. S. and Lii. C., Starch/Stärke, Vol. 44 (12) pp. 461-466 (1992).

Fermentation Product

The term "fermentation product" means a product produced by a process including a fermentation step using a fermenting organism. Fermentation products contemplated according to the invention include alcohols (e.g., ethanol, methanol, butanol); organic acids (e.g., citric acid, acetic acid, itaconic acid, lactic acid, gluconic acid); ketones (e.g., acetone); amino acids (e.g., glutamic acid); gases (e.g., H_2 and CO_2); antibiotics (e.g., penicillin and tetracycline); enzymes; vitamins (e.g., riboflavin, B_{12}, beta-carotene); and hormones. In a preferred embodiment the fermentation product is ethanol, e.g., fuel ethanol; drinking ethanol, i.e., potable neutral spirits; or industrial ethanol or products used in the consumable alcohol industry (e.g., beer and wine), dairy industry (e.g., fermented dairy products), leather industry and tobacco industry. Preferred beer types comprise ales, stouts, porters, lagers, bitters, malt liquors, happoushu, high-alcohol beer, low-alcohol beer, low-calorie beer or light beer. Preferred fermentation processes used include alcohol fermentation processes, as are well known in the art. Preferred fermentation processes are anaerobic fermentation processes, as are well known in the art.

Fermenting Organism

"Fermenting organism" refers to any organism, including bacterial and fungal organisms, suitable for use in a fermentation process and capable of producing desired a

fermentation product. Especially suitable fermenting organisms are able to ferment, i.e., convert, sugars, such as glucose or maltose, directly or indirectly into the desired fermentation product. Examples of fermenting organisms include fungal organisms, such as yeast. Preferred yeast includes strains of the *Saccharomyces* spp., and in particular, *Saccharomyces cerevisiae*. Commercially available yeast include, e.g., Red Star™/Lesaffre Ethanol Red (available from Red Star/Lesaffre, USA) FALI (available from Fleischmann's Yeast, a division of Burns Philp Food Inc., USA), SUPERSTART (available from Alltech), GERT STRAND (available from Gert Strand AB, Sweden) and FERMIOL (available from DSM Specialties).

Glucoamylase

The term "glucoamylase activity" means a glucan 1,4-alpha-glucosidase which hydrolyses the terminal 1,4-linked alpha-D-glucose residues successively from non-reducing ends of the chains with release of beta-D-glucose belonging to the Enzyme Class EC 3.2.1.3.

The glucoamylase used in a process of the invention has the amino acid sequence shown in SEQ ID NO: 2 (amino acid residues 1 to 561), or an amino acid sequence that is at least 70%, preferably at least 75%, or at least 80%, or at least 85%, or 90%, or at least 95%, at least 96%, at least 97%, at least 98% or even at least 99% identical to SEQ ID NO: 2 (amino acid residues 1 to 561). The glucoamylase derived from *Athelia rolfsii*, the amino acid sequence of which is available as SPTREMBL:Q12596, is almost identical to the one shown in SEQ ID NO: 2, except for one amino acid residue corresponding to the amino acid residue in position 97 of SEQ ID NO: 2, which in the database sequence is a serine, whereas in SEQ ID NO: 2 it is a proline. The annotation of the database sequence identifies amino acid residues 1-18 as a signal peptide, and residues 19-579 (i.e., 561 amino acid residues) as the mature glucoamylase enzyme, with residues 472-482 serving as a linker between the glucoamylase domain and the starch-binding domain comprised in residues 483-579.

The glucoamylase may in an embodiment be added in an amount of 0.001 to 10 AGU/g DS, preferably from 0.01 to 5 AGU/g DS, such as around 0.1, 0.3, 0.5, 1 or 2 AGU/g DS, especially 0.1 to 0.5 AGU/g DS or 0.02-20 AGU/g DS, preferably 0.1-10 AGU/g DS.

Alpha-Amylase

In a preferred embodiment an alpha-amylase may be added to the process of the invention. The alpha-amylase may according to the invention be of any origin. Preferred are alpha-amylases of fungal or bacterial origin.

In a preferred embodiment the alpha-amylase is an acid alpha-amylase, e.g., fungal acid alpha-amylase or bacterial acid alpha-amylase. The term "acid alpha-amylase" means an alpha-amylase (E.C. 3.2.1.1) which added in an effective amount has activity optimum at a pH in the range of 3 to 7, preferably from 3.5 to 6, or more preferably from 4-5.

Bacterial Alpha-Amylases

According to the invention the bacterial alpha-amylase may be derived from the genus *Bacillus*.

In a preferred embodiment the *Bacillus* alpha-amylase is derived from a strain of *B. licheniformis*, *B. amyloliquefaciens*, *B. subtilis* or *B. stearothermophilus*, but may also be derived from other *Bacillus* sp. Specific examples of contemplated alpha-amylases include the *Bacillus licheniformis* alpha-amylase (BLA) shown in SEQ ID NO: 5, the *Bacillus amyloliquefaciens* alpha-amylase (BAN) shown in SEQ ID NO: 6, and the *Bacillus stearothermophilus* alpha-amylase (BSG) shown in SEQ ID NO: 7. In an embodiment of the invention the alpha-amylase is an enzyme having a degree of identity of at least 60%, preferably at least 70%, more preferred at least 80%, even more preferred at least 90%, such as at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identity to any of the sequences shown in SEQ ID NO: 5, 6, or 7 of the present application or SEQ ID NO: 1, 2 or 3 in WO 99/19467.

The *Bacillus* alpha-amylase may also be a variant and/or hybrid, especially one described in any of WO 96/23873, WO 96/23874, WO 97/41213, WO 99/19467, WO 00/60059, and WO 02/10355 (all documents hereby incorporated by reference). Specifically contemplated alpha-amylase variants are disclosed in U.S. Patent Nos. 6,093,562, 6,187,576, and 6,297,038 (hereby incorporated by reference) and include *Bacillus stearothermophilus* alpha-amylase (BSG alpha-amylase) variants having a double deletion disclosed in WO 1996/023873 – see e.g., page 20, lines 1-10 (hereby incorporated by reference), preferably corresponding to delta(181-182) compared to the wild-type BSG alpha-amylase amino acid sequence set forth in SEQ ID NO: 7 disclosed in WO 99/19467 (hereby incorporated by reference). Even more preferred are *Bacillus* alpha-amylases, especially *Bacillus stearothermophilus* alpha-amylase, which have a double deletion corresponding to delta(181-182) and further comprise a N193F substitution (also denoted I181* + G182* + N193F) compared to the wild-type BSG alpha-amylase amino acid sequence set forth in SEQ ID NO: 7 of the present application and SEQ ID NO: 3 disclosed in WO 99/19467.

The alpha-amylase may also be a maltogenic alpha-amylase. A "maltogenic alpha-amylase" (glucan 1,4-alpha-maltohydrolase, E.C. 3.2.1.133) is able to hydrolyze amylose

and amylopectin to maltose in the alpha-configuration. A maltogenic alpha-amylase from *Bacillus stearothermophilus* strain NCIB 11837 is commercially available from Novozymes A/S, Denmark. The maltogenic alpha-amylase is described in U.S. Patent Nos. 4,598,048, 4,604,355 and 6,162,628, which are hereby incorporated by reference.

Bacterial Hybrid Alpha-Amylases

A hybrid alpha-amylase specifically contemplated comprises 445 C-terminal amino acid residues of the *Bacillus licheniformis* alpha-amylase (shown in SEQ ID NO: 5) and the 37 N-terminal amino acid residues of the alpha-amylase derived from *Bacillus amyloliquefaciens* (shown in SEQ ID NO: 6), with one or more, especially all, of the following substitution:

G48A+T49I+G107A+H156Y+A181T+N190F+I201F+A209V+Q264S

(using the *Bacillus licheniformis* numbering). Also preferred are variants having one or more of the following mutations (or corresponding mutations in other *Bacillus* alpha-amylase backbones): H154Y, A181T, N190F, A209V and Q264S and/or deletion of two residues between positions 176 and 179, preferably deletion of E178 and G179 (using the SEQ ID NO: 5 numbering of WO 99/19467).

The bacterial alpha-amylase may be added in amounts as are well-known in the art. When measured in KNU units (described below in the Materials & Methods"-section) the alpha-amylase activity is preferably present in an amount of 0.5-5,000 NU/g of DS, in an amount of 1-500 NU/g of DS, or more preferably in an amount of 5-1,000 NU/g of DS, such as 10-100 NU/g DS.

Fungal Alpha-Amylases

Fungal acid alpha-amylases include acid alpha-amylases derived from a strain of the genus *Aspergillus*, such as, *Aspergillus oryzae* and *Aspergillus niger* alpha-amylases.

A preferred acid fungal alpha-amylase is a Fungamyl-like alpha-amylase which is preferably derived from a strain of *Aspergillus oryzae*. In the present disclosure, the term "Fungamyl-like alpha-amylase" indicates an alpha-amylase which exhibits a high identity, i.e. more than 70%, more than 75%, more than 80%, more than 85% more than 90%, more than 95%, more than 96%, more than 97%, more than 98%, more than 99% or even 100% identity to the mature part of the amino acid sequence shown in SEQ ID NO: 10 in WO 96/23874 or shown as SEQ ID NO: 4 of the present application.

Another preferred acid alpha-amylase is derived from a strain *Aspergillus niger*. In a preferred embodiment the acid fungal alpha-amylase is the one from *A. niger* disclosed as "AMYA_ASPNG" in the Swiss-prot/TeEMBL database under the primary accession no.

P56271 and described in more detail in WO 89/01969 (Example 3). The acid *Aspergillus niger* acid alpha-amylase is also shown as SEQ ID NO: 3. Also variants of said acid fungal amylase having at least 70% identity, such as at least 80% or even at least 90% identity, such as at least 95%, 96%, 97%, 98%, or at least 99% identity to SEQ ID NO: 3 are contemplated. A commercially available acid fungal alpha-amylase derived from *Aspergillus niger* is SP288 (available from Novozymes A/S, Denmark).

The fungal acid alpha-amylase may also be a wild-type enzyme comprising a carbohydrate-binding module (CBM) and an alpha-amylase catalytic domain (i.e., none-hybrid), or a variant thereof. In an embodiment the wild-type acid alpha-amylase is derived from a strain of *Aspergillus kawachi*, in particular the alpha-amylase shown in SEQ ID NO: 31. Also variants of said fungal acid amylase having at least 70% identity, such as at least 80% or even at least 90% identity, such as at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to SEQ ID NO: 31 are contemplated.

Fungal Hybrid Alpha-Amylases

In a preferred embodiment the fungal acid alpha-amylase is a hybrid alpha-amylase. Preferred examples of fungal hybrid alpha-amylases include the ones disclosed in PCT/US2004/020499 (Novozymes), which is hereby incorporated by reference. A hybrid alpha-amylase may comprise an alpha-amylase catalytic domain (CD) and a carbohydrate-binding module (CBM) and optional a linker.

Hybrid enzymes or a genetically modified wild-type enzymes, as referred to herein, include species comprising an amino acid sequence of an alpha-amylase enzyme (EC 3.2.1.1) linked (i.e., covalently bound) to an amino acid sequence comprising a carbohydrate-binding module (CBM).

CBM-containing hybrid enzymes, as well as detailed descriptions of the preparation and purification thereof, are known in the art [see, e.g. WO 90/00609, WO 94/24158 and WO 95/16782, as well as Greenwood et al. Biotechnology and Bioengineering 44 (1994) pp. 1295-1305]. They may, e.g., be prepared by transforming into a host cell a DNA construct comprising at least a fragment of DNA encoding the carbohydrate-binding module ligated, with or without a linker, to a DNA sequence encoding the enzyme of interest, and growing the transformed host cell to express the fused gene. The resulting recombinant product (hybrid enzyme) - often referred to in the art as a "fusion protein - may be described by the following general formula:

A-CBM-MR-X

In the latter formula, A-CBM is the N-terminal or the C-terminal region of an amino acid sequence comprising at least the carbohydrate-binding module (CBM) *per se*. MR is the middle

region (the "linker"), and X is the sequence of amino acid residues of a polypeptide encoded by a DNA sequence encoding the enzyme (or other protein) to which the CBM is to be linked.

The moiety A may either be absent (such that A-CBM is a CBM *per se*, i.e. comprises no amino acid residues other than those constituting the CBM) or may be a sequence of one or more amino acid residues (functioning as a terminal extension of the CBM *per se*). The linker (MR) may be a bond, or a short linking group comprising from about 2 to about 100 carbon atoms, in particular of from 2 to 40 carbon atoms. However, MR is preferably a sequence of from about 2 to about 100 amino acid residues, more preferably of from 2 to 40 amino acid residues, such as from 2 to 15 amino acid residues.

The moiety X may constitute either the N-terminal or the C-terminal region of the overall hybrid enzyme.

It will thus be apparent from the above that the CBM in a hybrid enzyme of the type in question may be positioned C-terminally, N-terminally or internally in the hybrid enzyme.

Linker sequence

The optional linker sequence may be any suitable linker sequence. In preferred embodiments the linker sequence is derived from the *Athelia rolfsii* glucoamylase, the *A. niger* glucoamylase or the *A. kawachii* alpha-amylase such as a linker sequence selected from the group consisting of *A. niger* glucoamylase linker: TGGTTTTATPTGSGSVTSTSKTTATASKTSTSTSSTSA (SEQ ID NO: 8), *A. kawachii* alpha-amylase linker: TTTTTTAAATSTSKATTSSSSSSAAATTSSS (SEQ ID NO: 9), *Athelia rolfsii* glucoamylase linker: GATSPGGSSGS (SEQ ID NO: 10), and the PEPT linker: PEPTPEPT (SEQ ID NO: 11). In another preferred embodiment the hybrid enzymes has a linker sequence which differs from the amino acid sequence shown in SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, or SEQ ID NO: 11 in no more than 10 positions, no more than 9 positions, no more than 8 positions, no more than 7 positions, no more than 6 positions, no more than 5 positions, no more than 4 positions, no more than 3 positions, no more than 2 positions, or even no more than 1 position.

Carbohydrate-binding modules

A carbohydrate-binding module (CBM), or as often referred to, a carbohydrate-binding domain (CBD), is a polypeptide amino acid sequence which binds preferentially to a poly- or oligosaccharide (carbohydrate), frequently - but not necessarily exclusively - to a water-insoluble (including crystalline) form thereof.

CBMs derived from starch degrading enzymes are often referred to as starch-binding modules or SBMs (CBMs which may occur in certain amylolytic enzymes, such as certain

glucoamylases, or in enzymes such as cyclodextrin glucanotransferases, or in alpha-amylases). Likewise, other sub-classes of CBMs would embrace, e.g., cellulose-binding modules (CBMs from cellulolytic enzymes), chitin-binding modules (CBMs which typically occur in chitinases), xylan-binding modules (CBMs which typically occ,ur in xylanases), mannan-binding modules (CBMs which typically occur in mannanases). SBMs are often referred to as SBDs (Starch Binding Domains).

CBMs are found as integral parts of large polypeptides or proteins consisting of two or more polypeptide amino acid sequence regions, especially in hydrolytic enzymes (hydrolases) which typically comprise a catalytic module containing the active site for substrate hydrolysis and a carbohydrate-binding module (CBM) for binding to the carbohydrate substrate in question. Such enzymes can comprise more than one catalytic module and one, two or three CBMs, and optionally further comprise one or more polypeptide amino acid sequence regions linking the CBM(s) with the catalytic module(s), a region of the latter type usually being denoted a "linker". Examples of hydrolytic enzymes comprising a CBM - some of which have already been mentioned above - are cellulases, xylanases, mannanases, arabinofuranosidases, acetylesterases and chitinases. CBMs have also been found in algae, e.g., in the red alga *Porphyra purpurea* in the form of a non-hydrolytic polysaccharide-binding protein.

In proteins/polypeptides in which CBMs occur (e.g., enzymes, typically hydrolytic enzymes), a CBM may be located at the N or C terminus or at an internal position.

That part of a polypeptide or protein (e.g., hydrolytic enzyme) which constitutes a CBM *per se* typically consists of more than about 30 and less than about 250 amino acid residues.

The "Carbohydrate-Binding Module of Family 20" or a CBM-20 module is in the context of this invention defined as a sequence of approximately 100 amino acids having at least 45% identity to the Carbohydrate-Binding Module (CBM) of the polypeptide disclosed in figure 1 by Joergensen et al (1997) in Biotechnol. Lett. 19:1027-1031. The CBM comprises the last 102 amino acids of the polypeptide, i.e., the subsequence from amino acid 582 to amino acid 683. The numbering of Glycoside Hydrolase Families applied in this disclosure follows the concept of Coutinho, P.M. & Henrissat, B. (1999) *CAZy - Carbohydrate-Active Enzymes server* at URL: afmb.cnrs-mrs.fr/~cazy/CAZY/index.html or alternatively Coutinho, P.M. & Henrissat, B. 1999; The modular structure of cellulases and other carbohydrate-active enzymes: an integrated database approach. In *"Genetics, Biochemistry and Ecology of Cellulose Degradation"*, K. Ohmiya, K. Hayashi, K. Sakka, Y. Kobayashi, S. Karita and T. Kimura eds., Uni Publishers Co., Tokyo, pp. 15-23, and Bourne, Y. & Henrissat, B. 2001;

Glycoside hydrolases and glycosyltransferases: families and functional modules, *Current Opinion in Structural Biology* 11:593-600.

Examples of enzymes which comprise a CBM suitable for use in the context of the invention are alpha-amylases, maltogenic alpha-amylases, cellulases, xylanases, mannanases, arabinofuranosidases, acetylesterases and chitinases. Further CBMs of interest in relation to the present invention include CBMs deriving from glucoamylases (EC 3.2.1.3) or from CGTases (EC 2.4.1.19).

CBMs deriving from fungal, bacterial or plant sources will generally be suitable for use in the context of the invention. Preferred are CBMs of fungal origin, more preferably from *Aspergillus* sp., *Bacillus* sp., *Klebsiella* sp., or *Rhizopus* sp. In this connection, techniques suitable for isolating the relevant genes are well known in the art.

Preferred for the invention is CBMs of Carbohydrate-Binding Module Family 20. CBMs of Carbohydrate-Binding Module Family 20 suitable for the invention may be derived from glucoamylases of *Aspergillus awamori* (SWISSPROT Q12537), *Aspergillus kawachii* (SWISSPROT P23176), *Aspergillus niger* (SWISSPROT P04064), *Aspergillus oryzae* (*SWISSPROT* P36914), from alpha-amylases of *Aspergillus kawachii* (EMBL:#AB008370), *Aspergillus nidulans* (NCBI AAF17100.1), from beta-amylases of *Bacillus cereus* (SWISSPROT P36924), or from CGTases of *Bacillus circulans* (SWISSPROT P43379). Preferred is a CBM from the alpha-amylase of *Aspergillus kawachii* (EMBL:#AB008370) as well as CBMs having at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95, at least 96%, at least 97%, at least 98% or even at least 99% identity to the CBM of the alpha-amylase of *Aspergillus kawachii* (EMBL:#AB008370), i.e., a CBM having at least 50%, 60%, 70%, 80%, 95%, 96%, 97%, 98% or even at least 99% identity to the amino acid sequence of SEQ ID NO: 12. Also preferred for the invention are the CBMs of Carbohydrate-Binding Module Family 20 having the amino acid sequences shown in SEQ ID NO: 14 (*Bacillus flavothermus* CBM), SEQ ID NO: 15 (*Bacillus* sp. CBM), and SEQ ID NO: 16 (*Alcaliphilic Bacillus* CBM) and disclosed in International application PCT/DK2004/000456 as SEQ ID NO: 1, SEQ ID NO: 2 and SEQ ID NO: 3 respectively. Further preferred CBMs include the CBMs of the glucoamylase from *Hormoconis* sp. such as from *Hormoconis resinae* (Syn. Creosote fungus or *Amorphotheca resinae*) such as the CBM of SWISSPROT:Q03045 (SEQ ID NO: 17), from *Lentinula* sp. such as from *Lentinula edodes* (shiitake mushroom) such as the CBM of SPTREMBL:Q9P4C5 (SEQ ID NO: 18), from *Neurospora* sp. such as from *Neurospora crassa* such as the CBM of SWISSPROT:P14804 (SEQ ID NO: 19), from *Talaromyces* sp. such as from *Talaromyces byssochlamydioides* such as the CBM of NN005220 (SEQ ID NO: 20), from *Geosmithia* sp. such as from *Geosmithia cylindrospora*, such as the CBM of NN48286 (SEQ ID NO: 21), from *Scorias* sp.

such as from *Scorias spongiosa* such as the CBM of NN007096 (SEQ ID NO: 22), from *Eupenicillium* sp. such as from *Eupenicillium ludwigii* such as the CBM of NN005968 (SEQ ID NO: 23), from *Aspergillus* sp. such as from *Aspergillus japonicus* such as the CBM of NN001136 (SEQ ID NO: 24), from *Penicillium* sp. such as from *Penicillium* cf. *miczynskii* such as the CBM of NN48691 (SEQ ID NO: 25), from Mz1 *Penicillium* sp. such as the CBM of NN48690 (SEQ ID NO: 26), from *Thysanophora* sp. such as the CBM of NN48711 (SEQ ID NO: 27), and from *Humicola* sp. such as from *Humicola grisea var. thermoidea* such as the CBM of SPTREMBL:Q12623 (SEQ ID NO: 28). Most preferred CBMs include the CBMs of the glucoamylase from *Aspergillus* sp. such as from *Aspergillus niger,* such as SEQ ID NO: 29, and *Athelia* sp. such as from *Athelia rolfsii,* such as SEQ ID NO: 30.

Preferably the hybrid enzyme comprises a CBM sequence having at least 50%, at least 60%, at least 70%, at least 80% at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or even at least 99% identity to any of the amino acid sequences shown in SEQ ID NO: 13, SEQ ID NO: 14 10, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29 or SEQ ID NO: 30. In yet another preferred embodiment the CBM sequence has an amino acid sequence which differs from the amino acid sequence amino acid sequence shown in SEQ ID NO: 13, SEQ ID NO: 14 10, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29 or SEQ ID NO; 30 in no more than 10 amino acid positions, no more than 9 positions, no more than 8 positions, no more than 7 positions, no more than 6 positions, no more than 5 positions, no more than 4 positions, no more than 3 positions, no more than 2 positions, or even no more than 1 position. In a most preferred embodiment the hybrid enzyme comprises a CBM derived from a glucoamylase from *Athelia rolfsii*, such as the glucoamylase from *A. rolfsii* AHU 9627 described in U.S. Patent No. 4,727,026.

Further suitable CBMs of Carbohydrate-Binding Module Family 20 may be found at URL: afmb.cnrs-mrs.fr/~cazy/CAZY/index.html).

Once a nucleotide sequence encoding the substrate-binding (carbohydrate-binding) region has been identified, either as cDNA or chromosomal DNA, it may then be manipulated in a variety of ways to fuse it to a DNA sequence encoding the enzyme of interest. The DNA fragment encoding the carbohydrate-binding amino acid sequence, and the DNA encoding the enzyme of interest are then ligated with or without a linker. The resulting ligated DNA may then be manipulated in a variety of ways to achieve expression.

Catalytic domain in hybrid

Alpha-amylases (in particular acid alpha-amylases) which are appropriate as the basis for CBM/amylase hybrids of the types employed in the context of the present invention include those of fungal origin. Preferred examples are the ones described above in the "fungal alpha-amylases"-section, which include acid alpha-amylases from *Aspergillus niger* shown as SEQ ID NO: 3 or *Aspergillus oryzae* shown in SEQ ID NO: 4.

Even more preferred is an embodiment wherein the hybrid enzyme comprises an alpha-amylase sequence derived from the *A. oryzae* acid alpha-amylase (Fungamyl™, SEQ ID NO: 4), and/or a linker sequence derived from the *A. kawachii* alpha-amylase (SEQ ID NO: 9 or the *A. rolfsii* glucoamylase (SEQ ID NO: 10), and/or a CBM derived from the *A. kawachii* alpha-amylase (SEQ ID NO: 13) or the *A. rolfsii* glucoamylase (SEQ ID NO: 30).

Also preferred is an embodiment wherein the hybrid enzyme comprises an alpha-amylase sequence derived from the *A. niger* acid alpha-amylase (SP288) catalytic module having the sequence shown in SEQ ID NO: 3, and/or a linker sequence derived from the *A. kawachii* alpha-amylase (SEQ ID NO: 9) or the *A. rolfsii* glucoamylase (SEQ ID NO: 10) , and/or the CBM derived from the *A. kawachii* alpha-amylase (SEQ ID NO: 12), the *A. rolfsii* glucoamylase (SEQ ID NO: 30) or the *A. niger* glucoamylase (SEQ ID NO: 29). In a particularly preferred embodiment the hybrid enzyme comprises the *A. niger* acid alpha-amylase (SP288) catalytic module having the sequence shown in SEQ ID NO: 3 and the *A. kawachii* alpha-amylase linker (SEQ ID NO: 9) and CBM (SEQ ID NO: 12).

In a specific embodiment the hybrid enzyme is the mature part of the amino acid sequence shown in SEQ ID NO: 33 (*A. niger* acid alpha-amylase catalytic domain-*A. kawachii* alpha-amylase linker-*A. niger* glucoamylase CBM), SEQ ID NO: 35 (*A. niger* acid alpha-amylase catalytic domain-*A. kawachii* alpha-amylase linker-*A. rolfsii* glucoamylase CBM), or SEQ ID NO: 37 (*A. oryzae* acid alpha-amylase catalytic domain-*A. kawachii* alpha-amylase linker-*A. kawachii* alpha-amylase CBM), or SEQ ID NO: 39 (*A. niger* acid alpha-amylase catalytic domain-*A. rolfsii* glucoamylase linker-*A. rolfsii* glucoamylase CBM), or SEQ ID NO: 41 (*A. oryzae* acid alpha-amylase catalytic domain-*A. rolfsii* glucoamylase linker-*A. rolfsii* glucoamylase CBM) or the hybrid consisting of *A. niger* acid alpha-amylase catalytic domain (SEQ ID NO: 3)-*A. kawachii* alpha-amylase linker (SEQ ID NO: 9) - *A. kawachii* alpha-amylase CBM (SEQ ID NO: 13) or a hybrid enzyme that has an amino acid sequence having at least 50%, at least 60%, at least 70%, at least 80%, or even at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to any of the afore mentioned amino acid sequences.

In another preferred embodiment the hybrid enzyme has an amino acid sequence which differs from the amino acid sequence amino acid sequence shown in SEQ ID NO: 33

(*A. niger* acid alpha-amylase catalytic domain-*A. kawachii* alpha-amylase linker-*A. niger* glucoamylase CBM), SEQ ID NO: 35 (*A. niger* acid alpha-amylase catalytic domain-*A. kawachii* alpha-amylase linker-*A. rolfsii* glucoamylase CBM), SEQ ID NO: 37 (*A. oryzae* acid alpha-amylase catalytic domain-*A. kawachii* alpha-amylase linker-*A. kawachii* alpha-amylase CBM) , SEQ ID NO: 39 (*A. niger* acid alpha-amylase catalytic domain-*A. rolfsii* glucoamylase linker-*A. rolfsii* glucoamylase CBM) or SEQ ID NO: 41 (*A. oryzae* acid alpha-amylase catalytic domain-*A. rolfsii* glucoamylase linker-*A. rolfsii* glucoamylase CBM) or the hybrid consisting of *A. niger* acid alpha-amylase catalytic domain (SEQ ID NO: 3)-*A. kawachii* alpha-amylase linker (SEQ ID NO: 9) -*A. kawachii* alpha-amylase CBM (SEQ ID NO: 13) in no more than 10 positions, no more than 9 positions, no more than 8 positions, no more than 7 positions, no more than 6 positions, no more than 5 positions, no more than 4 positions, no more than 3 positions, no more than 2 positions, or even no more than 1 position.

Commercial Alpha-Amylase Products

Preferred commercial compositions comprising alpha-amylase include MYCOLASE from DSM (Gist Brocades), BAN™, TERMAMYL™ SC, FUNGAMYL™, LIQUOZYME™ X and SAN™ SUPER, SAN™ EXTRA L (Novozymes A/S) and CLARASE™ L-40,000, DEX-LO™, SPEYME FRED, SPEZYME™ AA, and SPEZYME™ DELTA AA (Genencor Int.), and the acid fungal alpha-amylase sold under the trade name SP288 (available from Novozymes A/S, Denmark).

The acid alpha-amylases may according to the invention be added in an amount of 0.1 to 10 AFAU/g DS, preferably 0.10 to 5 AFAU/g DS, especially 0.3 to 2 AFAU/g DS.

Combination of glucoamylase and acid alpha-amylase

Even though the presence of acid alpha-amylase is not mandatory according to the invention the activities of acid alpha-amylase and glucoamylase may be present in a ratio of between 0.3 and 5.0 AFAU/AGU. More preferably the ratio between acid alpha-amylase activity and glucoamylase activity is at least 0.35, at least 0.40, at least 0.50, at least 0.60, at least 0.7, at least 0.8, at least 0.9, at least 1.0, at least 1.1, at least 1.2, at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.85, or even at least 1.9 AFAU/AGU. However, the ratio between acid alpha-amylase activity and glucoamylase activity should preferably be less than 4.5, less than 4.0, less than 3.5, less than 3.0, less than 2.5, or even less than 2.25 AFAU/AGU. In AUU/AGI the activities of acid alpha-amylase and glucoamylase are preferably present in a ratio of between 0.4 and 6.5 AUU/AGI. More preferably the ratio between acid alpha-amylase activity and glucoamylase activity is at least 0.45, at least 0.50, , at least 0.60, at least 0.7, at least 0.8, at least 0.9, at least 1.0, at least

1.1, at least 1.2, at least 1.3, at least 1.4, at least 1.5, at least 1.6, at least 1.7, at least 1.8, at least 1.9, at least 2.0, at least 2.1, at least 2.2, at least 2.3, at least 2.4, or even at least 2.5 AUU/AGI. However, the ratio between acid alpha-amylase activity and glucoamylase activity is preferably less than 6.0, less than 5.5, less than 4.5, less than 4.0, less than 3.5, or even less than 3.0 AUU/AGI.

Protease

According to the process of the invention a protease may be present during saccharification and/or fermentation as well.

In a preferred embodiment the protease is an acid protease of microbial origin, preferably of fungal or bacterial origin.

Suitable proteases include microbial proteases, such as fungal and bacterial proteases. Preferred proteases are acidic proteases, i.e., proteases characterized by the ability to hydrolyze proteins under acidic conditions below pH 7.

Contemplated acid fungal proteases include fungal proteases derived from Aspergillus, *Mucor, Rhizopus, Candida, Coriolus, Endothia, Enthomophtra, Irpex, Penicillium, Sclerotiumand Torulopsis.* Especially contemplated are proteases derived from *Aspergillus niger* (see, e.g., Koaze et al., (1964), Agr. Biol. Chem. Japan, 28, 216), *Aspergillus saitoi* (see, e.g., Yoshida, (1954) J. Agr. Chem. Soc. Japan, 28, 66), *Aspergillus awamori* (Hayashida et al., (1977) Agric. Biol. Chem., 42(5), 927-933, *Aspergillus aculeatus* (WO 95/02044), or *Aspergillus oryzae,* such as the pepA protease; and acidic proteases from *Mucor pusillus* or *Mucor miehei.*

Contemplated are also neutral or alkaline proteases, such as a protease derived from a strain of *Bacillus.* A particular protease contemplated for the invention is derived from *Bacillus amyloliquefaciens* and has the sequence obtainable at Swissprot as Accession No. P06832. Also contemplated are the proteases having at least 90% identity to amino acid sequence obtainable at Swissprot as Accession No. P06832 such as at least 92%, at least 95%, at least 96%, at least 97%, at least 98%, or particularly at least 99% identity.

Further contemplated are the proteases having at least 90% identity to amino acid sequence disclosed as SEQ.ID.NO:1 in the WO 2003/048353 such as at 92%, at least 95%, at least 96%, at least 97%, at least 98%, or particularly at least 99% identity.

Also contemplated are papain-like proteases such as proteases within E.C. 3.4.22.* (cysteine protease), such as EC 3.4.22.2 (papain), EC 3.4.22.6 (chymopapain), EC 3.4.22.7 (asclepain), EC 3.4.22.14 (actinidain), EC 3.4.22.15 (cathepsin L), EC 3.4.22.25 (glycyl endopeptidase) and EC 3.4.22.30 (caricain).

Proteases may be added in the amounts of 0.1-1000 AU/kg dm, preferably 1-100 AU/kg DS and most preferably 5-25 AU/kg DS.

Additional Ingredients

Additional ingredients may be present during saccharification and/or fermentation to increase the effectiveness of the process of the invention. For instance, nutrients (e.g. fermentation organism micronutrients), antibiotics, salts (e.g., zinc or magnesium salts), other enzymes such as phytase, cellulase, hemicellulase, exo and endoglucanase, and xylanases.

Recovery of fermentation product

The fermentation product, such as ethanol, may optionally be recovered after fermentation. The recovery may be performed by any conventional manner such as, e.g., distillation.

The invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed, since these embodiments are intended as illustrations of several aspects of the invention. Any equivalent embodiments are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims. In the case of conflict, the present disclosure including definitions will control.

Various references are cited herein, the disclosures of which are incorporated by reference in their entireties.

MATERIALS AND METHODS

Glucoamylases:

* Glucoamylase derived from *Athelia rolfsii* disclosed in SEQ ID NO: 2 and available from Novozymes A/S.

* Glucoamylase derived from *Aspergillus niger* disclosed in Boel et al. (1984), EMBO J. 3 (5) p. 1097-1102 and available from Novozymes A/S.

* Glucoamylase derived from *Talaromyces emersonii* disclosed in WO 99/28448 and available from Novozymes A/S.

* Acid fungal alpha-amylase is derived from *Aspergillus niger* consisting of the *Aspergillus niger* acid alpha-amylase catalytic domain (SEQ ID NO: 3), *Aspergillus kawachii* alpha-amylase linker (SEQ ID NO: 9) –*Aspergillus kawachii* alpha-amylase CBM (SEQ ID NO: 13).

Yeast: Red Star™ available from Red Star/Lesaffre, USA

Homology/identity

In context of the present invention "homology" means the degree of identity between two amino acid sequences. The homology may suitably be determined by computer programs known in the art, such as, GAP provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-453. The following settings for polypeptide sequence comparison are used: GAP creation penalty of 3.0 and GAP extension penalty of 0.1.

Alpha-amylase activity (KNU)

The amylolytic activity may be determined using potato starch as substrate. This method is based on the break-down of modified potato starch by the enzyme, and the reaction is followed by mixing samples of the starch/enzyme solution with an iodine solution. Initially, a blackish-blue color is formed, but during the break-down of the starch the blue color gets weaker and gradually turns into a reddish-brown, which is compared to a colored glass standard.

One Kilo Novo alpha amylase Unit (KNU) is defined as the amount of enzyme which, under standard conditions (i.e. at 37°C +/- 0.05; 0.0003 M Ca^{2+}; and pH 5.6) dextrinizes 5260 mg starch dry substance Merck Amylum solubile.

A folder EB-SM-0009.02/01 describing this analytical method in more detail is available upon request to Novozymes A/S, Denmark, which folder is hereby incorporated by reference.

Acid alpha-amylase activity

When used according to the present invention the activity of any acid alpha-amylase may be measured in AFAU (Acid Fungal Alpha-amylase Units). Alternatively activity of acid alpha-amylase may be measured in AAU (Acid Alpha-amylase Units).

Acid Alpha-amylase Units (AAU)

The acid alpha-amylase activity can be measured in AAU (Acid Alpha-amylase Units), which is an absolute method. One Acid Amylase Unit (AAU) is the quantity of enzyme converting 1 g of starch (100% of dry matter) per hour under standardized conditions into a

product having a transmission at 620 nm after reaction with an iodine solution of known strength equal to the one of a color reference.

Standard conditions/reaction conditions:

Substrate:	Soluble starch. Concentration approx. 20 g DS/L.
Buffer:	Citrate, approx. 0.13 M, pH=4.2
Iodine solution:	40.176 g potassium iodide + 0.088 g iodine/L
City water	15°-20°dH (German degree hardness)
pH:	4.2
Incubation temperature:	30°C
Reaction time:	11 minutes
Wavelength:	620 nm
Enzyme concentration:	0.13-0.19 AAU/mL
Enzyme working range:	0.13-0.19 AAU/mL

The starch should be Lintner starch, which is a thin-boiling starch used in the laboratory as colorimetric indicator. Lintner starch is obtained by dilute hydrochloric acid treatment of native starch so that it retains the ability to color blue with iodine. Further details can be found in European Patent No. 140410, which disclosure is hereby incorporated by reference.

Acid alpha-amylase activity (AFAU)

Acid alpha-amylase activity may be measured in AFAU (Acid Fungal Alpha-amylase Units), which are determined relative to an enzyme standard. 1 FAU is defined as the amount of enzyme which degrades 5.260 mg starch dry matter per hour under the below mentioned standard conditions.

Acid alpha-amylase, an endo-alpha-amylase (1,4-alpha-D-glucan-glucanohydrolase, E.C. 3.2.1.1) hydrolyzes alpha-1,4-glucosidic bonds in the inner regions of the starch molecule to form dextrins and oligosaccharides with different chain lengths. The intensity of color formed with iodine is directly proportional to the concentration of starch. Amylase activity is determined using reverse colorimetry as a reduction in the concentration of starch under the specified analytical conditions.

$$\text{STARCH} + \text{IODINE} \xrightarrow[40°,\ pH\ 2,5]{\text{ALPHA - AMYLASE}} \text{DEXTRINS} + \text{OLIGOSACCHARIDES}$$

$\lambda = 590$ nm

blue/violet t = 23 sec. decoloration

Standard conditions/reaction conditions:

Substrate:	Soluble starch, approx. 0.17 g/L
Buffer:	Citrate, approx. 0.03 M
Iodine (I2):	0.03 g/L
$CaCl_2$:	1.85 mM
pH:	2.50 ± 0.05
Incubation temperature:	40°C
Reaction time:	23 seconds
Wavelength:	590nm
Enzyme concentration:	0.025 AFAU/mL
Enzyme working range:	0.01-0.04 AFAU/mL

A folder EB-SM-0259.02/01 describing this analytical method in more detail is available upon request to Novozymes A/S, Denmark, which folder is hereby incorporated by reference.

Glucoamylase activity

Glucoamylase activity may be measured in AGI units or in AmyloGlucosidase Units (AGU).

Glucoamylase activity (AGI)

Glucoamylase (equivalent to amyloglucosidase) converts starch into glucose. The amount of glucose is determined here by the glucose oxidase method for the activity determination. The method described in the section 76-11 Starch—Glucoamylase Method with Subsequent Measurement of Glucose with Glucose Oxidase in "Approved methods of the American Association of Cereal Chemists". Vol.1-2 AACC, from American Association of Cereal Chemists, (2000); ISBN: 1-891127-12-8.

One glucoamylase unit (AGI) is the quantity of enzyme which will form 1 micromol of glucose per minute under the standard conditions of the method.

Standard conditions/reaction conditions:

Substrate:	Soluble starch, concentration approx. 16 g dry matter/L.
Buffer:	Acetate, approx. 0.04 M, pH=4.3
pH:	4.3
Incubation temperature:	60°C
Reaction time:	15 minutes
Termination of the reaction:	NaOH to a concentration of approximately 0.2 g/L (pH~9)
Enzyme concentration:	0.15-0.55 AAU/mL.

The starch should be Lintner starch, which is a thin-boiling starch used in the laboratory as colorimetric indicator. Lintner starch is obtained by dilute hydrochloric acid treatment of native starch so that it retains the ability to color blue with iodine.

Glucoamylase activity (AGU)

The Novo Glucoamylase Unit (AGU) is defined as the amount of enzyme, which hydrolyzes 1 micromole maltose per minute under the standard conditions 37°C, pH 4.3, substrate: maltose 23.2 mM, buffer: acetate 0.1 M, reaction time 5 minutes.

An autoanalyzer system may be used. Mutarotase is added to the glucose dehydrogenase reagent so that any alpha-D-glucose present is turned into beta-D-glucose. Glucose dehydrogenase reacts specifically with beta-D-glucose in the reaction mentioned above, forming NADH which is determined using a photometer at 340 nm as a measure of the original glucose concentration.

AMG incubation:

Substrate:	maltose 23.2 mM
Buffer:	acetate 0.1 M
pH:	4.30 ± 0.05
Incubation temperature:	37°C ± 1
Reaction time:	5 minutes
Enzyme working range:	0.5-4.0 AGU/mL

Color reaction:

GlucDH:	430 U/L
Mutarotase:	9 U/L
NAD:	0.21 mM

Buffer:	phosphate 0.12 M; 0.15 M NaCl
pH:	7.60 ± 0.05
Incubation temperature:	37°C ± 1
Reaction time:	5 minutes
Wavelength:	340 nm

A folder (EB-SM-0131.02/01) describing this analytical method in more detail is available on request from Novozymes A/S, Denmark, which folder is hereby incorporated by reference.

Proteolytic Activity (AU)

The proteolytic activity may be determined with denatured hemoglobin as substrate. In the Anson-Hemoglobin method for the determination of proteolytic activity denatured hemoglobin is digested, and the undigested hemoglobin is precipitated with trichloroacetic acid (TCA). The amount of TCA soluble product is determined with phenol reagent, which gives a blue color with tyrosine and tryptophan.

One Anson Unit (AU) is defined as the amount of enzyme which under standard conditions (i.e. 25°C, pH 7.5 and 10 min. reaction time) digests hemoglobin at an initial rate such that there is liberated per minute an amount of TCA soluble product which gives the same color with phenol reagent as one milliequivalent of tyrosine.

A folder AF 4/5 describing the analytical method in more detail is available upon request to Novozymes A/S, Denmark, which folder is hereby incorporated by reference.

Example 1

Evaluation of *Athelia rolfsii* glucoamylase in 'One-Step' Fuel Ethanol Fermentations

The relative performance of *Athelia rolfsii* glucoamylase to *Aspergillus niger* glucoamylase and *Talaromyces emersonii* glucoamylase was evaluated via mini-scale fermentations. About 380 g of milled corn (ground in a pilot scale hammer mill through a 1.65 mm screen) was added to about 620 g tap water. This mixture was supplemented with 3 mL 1 g/L penicillin. The pH of this slurry was adjusted to 5.0 with 40% H_2SO_4. The dry solid (DS) level was determined in triplicate to be about 32%. Approximately 5 g of this slurry was added to 15 mL tubes.

A two dose dose-response was conducted with each enzyme. Dosages used were 0. 3 and 0. 6 nmol/ g DS. Six replicates of each treatment were run.

After dosing the tubes were inoculated with 0.04 mL/g mash of yeast propagate (Red Star™ yeast) that had been grown for 22.5 hours on corn mash. Tubes were capped with a screw on top which had been punctured with a small needle to allow gas release and vortexed briefly before weighing and incubation at 32°C. 70 hours fermentations were carried out and ethanol yields were determined by weighing the tubes. Tubes were vortexed briefly before weighing. The result of the experiment is shown in Table 1.

It can be seen from Table 1 the ethanol yield per gram DS is significantly higher when using the *Athelia rolfsii* glucoamylase compared to yields for the wild-type *Aspergillus niger* and *Talaromyces* emersonii glucoamylases.

Table 1

Glucoamylase	nmol /g DS	Ethanol yields
Athelia rolfsii	0. 3	96.4
Aspergillus niger		47.2
Talaromyces emersonii		30.5
Athelia rolfsii	0. 6	121.9
Aspergillus niger		87.2
Talaromyces emersonii		43.4

Example 2

Evaluation of *Athelia rolfsii* glucoamylase in combination with acid fungal alpha-amylase in 'One-Step' Saccharification

The glucose concentration after one step saccharification with *Athelia rolfsii* glucoamylase alone and in combination with a fungal acid alpha-amylase activity (*Aspergillus niger* acid alpha-amylase hybrid with starch-binding domain from *Aspergillus kawachii* alpha-amylase), respectively, was compared with *Aspergillus niger* glucoamylase alone and in combination the same fungal acid alpha-amylase under the same conditions and at the same dose levels.

The evaluation was made by mini-scale saccharification very similar to the mini-scale fermentation used in Example 1, except for the fact that no yeast was added and a buffer was used to hold the pH at 4.5.

Briefly, 194 g of milled corn was mixed with 306 g of 37 mM NaOAc, 0.025% sodium azide, 20 mM $CaCl_2$, pH 4.5 to yield a slurry of approximately 35% DS. The pH of this slurry was adjusted to 4.5 with 40% H_2SO_4 (initial pH, before adjustment, was around 4.9). The slurry was allowed to hydrate while stirring at room temperature for one hour. Approximately 5 g of this slurry was added to a 20 mL vial for each reaction. The vials containing corn slurry were then pre-incubated at 32°C for one hour prior to dosing. Each vial was dosed with the

appropriate amount of enzyme, capped and vortexed immediately. Actual dosages were based on the exact weight of corn slurry in each vial. Three replicates were run for each reaction. Vials were incubated at 32°C. Each vial was vortexed after 4 hours and the reactions were stopped by addition of 50 microL of 40% H_2SO_4 and prepped for HPLC analysis. The HPLC preparation consisted of centrifuging, and filtering through a 0.45 micro m filter. Samples awaiting HPLC analysis were stored at 4°C.

Table 2 shows the glucose concentration after 4 hours of saccharification.

Treatment	4 hour Glucose (g/L)
0.263 mg/g DS *Aspergillus niger* glucoamylase	33.2
0.263 mg/g DS *Aspergillus niger* glucoamylase + 0.034 mg/g DS *Aspergillus niger* acid alpha-amylase with *Aspergillus kawachii* alpha-amylase linker and CBM	40.4
0.263 mg/g DS *Athelia rolfsii* glucoamylase	45.6
0.263 mg/g DS *Athelia rolfsii* glucoamylase + 0.034 mg/g DS *Aspergillus niger* acid alpha-amylase with *Aspergillus kawachii* alpha-amylase linker and CBM	63.2

The obtained glucose level after saccharification correlates with the ethanol fermentation yield that would be obtained if fermented by *Saccharomyces* yeast. Consequently, the above results show that *Athelia rolfsii* glucoamylase alone and in combination with a fungal acid alpha-amylase performs better than *Aspergillus niger* glucoamylase alone and in combination with a fungal acid alpha-amylase under the same conditions.